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TITLE: Targeting L-Selectin to Improve Neurologic and Urologic Function After Spinal Cord Injury

PRINCIPAL INVESTIGATOR: Linda Noble

CONTRACTING ORGANIZATION: University of California at San Francisco,
San Francisco, CA 941186215

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14. ABSTRACT

Purpose: We are evaluating the efficacy of diclofenac (DFA), an anti-inflammatory agent with L-Selectin sheddase activity, in a murine model of spinal cord injury.

Scope: These studies have focused on the efficacy of DFA in the context of dose, optimal therapeutic window, and dependency on injury severity, using clinically relevant outcome measures that include neurologic assessments and assays of bladder function.

Major findings:

-Mice with spinal cord injury (SCI) were administered a range of doses of DFA (60mg/kg, 40mg/kg, 20mg/kg, 10mg/kg, 5mg/kg, 1mg/kg, or vehicle) and L-selectin sheddase activity was monitored at 8 hours, 1 day, 3 days, and 7 days using ELISA. 40mg/kg and 60mg/kg DFA were the minimally effective doses to induce L-selectin sheddase activity, which was detected at 8 and 24 hours.

Mice were immediately administered vehicle, 40mg/kg DFA, or 60mg/kg DFA after a mild SCI. All mice showed similar marked neurological improvement over time, based upon traditional assessments of neurological function (coordination and kinematics), consistent with the natural recovery that accompanies mild forms of SCI. Importantly, neurologic recovery was improved in mice receiving 40mg/kg, based upon assessment of the percentage of mice frequently/consistently plantar stepping. These encouraging findings not only highlight efficacy of DFA at 40mg/kg after SCI, but reveal a sensitive assay for recovery of function after mild injury that is critical to the spinal cord injured patient; namely the ability to plantar step. Subsequent studies will utilize this assay as well as standard assays of neurological function to assess efficacy in paradigms of moderate and severe injury..

Significance: We can reliably monitor L-selectin sheddase activity in response to DFA administration. This will allow for the robust detection of L-selectin sheddase activity during Task 4a of Specific Aim 4. We identified the minimal effective dose of DFA, 40mg/kg, which induces L-selectin shedding. Utilizing this dose, we demonstrated neurologic recovery after immediate administration to mice receiving mild SCI, using an assay that critically assesses the ability to step. This finding, together with earlier results showing efficacy of 40mg/kg DFO after a moderate SCI, reinforce the candidacy of DFA as a therapeutic for the spinal cord injured patient. Given these successes, we are optimally positioned to proceed with Specific Aim 2, where we will evaluate the window and duration of efficacy after a moderate SCI. Finally, we have established protocols for assessing urological function that will be utilized for Specific Aim 3.

15. SUBJECT TERMS

spinal cord injury, L-Selectin, diclofenac, mouse, urologic function, neurologic function

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INTRODUCTION

This proposal is investigating the hypothesis that the anti-inflammatory drug diclofenac (DFA), acting as an L-selectin sheddase, will improve neurologic outcome and ameliorate neurogenic bladder dysfunction resulting from spinal cord injury (SCI). L-selectin is expressed on the surface of all leukocytes. Preliminary data using the L-selectin knockout (KO) mouse confirmed the dependency of L-selectin on neurologic recovery and thus served as the basis for pharmacologic targeting of this molecule in a murine model of SCI. The specific aims of this proposal are to define the minimal effective dose of DFA, the optimal window of therapeutic intervention for DFA, whether DFA administration improves bladder function, and if the efficacy of DFA is dependent on proteolytic cleavage of L-selectin.

Please note that each task is indicated in bold. We also requested a change to our tasks related to Specific Aim 1. These requested changes are indicated in bold italics.

BODY

Specific Aim 1

Task 1. Define the minimal effective dose of DFA

1a. Obtain animal use protocol approval (months 1-4)

We received approval from the UCSF IACUC and ACURO to conduct these studies.

1b. Assay L-Selectin sheddase activity in both blood and spinal cord by flow cytometry at 8 hours to 7 days after a single bolus administration of DFA (at 1, 5, 10, 20, or 40 mg/kg) given immediately after injury (months 5-9)

We first tested the feasibility of assaying L-selectin sheddase activity by flow cytometry vs. utilizing an enzyme-linked immunosorbent assay (ELISA) specific to soluble L-Selectin. Male C57BL/6 mice were subjected to a 2g weight dropped 7.5 cm onto the exposed spinal cord at the thoracic 9 vertebral level (moderate injury). DFA (40 mg/kg) or vehicle was administered immediately following spinal cord injury (SCI) (n=10/group). Plasma and spinal cord tissue was collected 1 day post-SCI/DFA administration and processed by either flow cytometry (n=5/group) or ELISA (n=5/group). Data from plasma samples processed by flow cytometry (Figure 1), from plasma samples processed by ELISA (Figure 2), from spinal cord samples processed by flow cytometry (Figure 3), and from spinal cord samples processed by ELISA (Figure 4), are below. Significance was defined as $p < 0.05$ and determined using a one-way ANOVA followed by a Dunnett's post-hoc test for flow data or a one-tailed t-test for ELISA data.

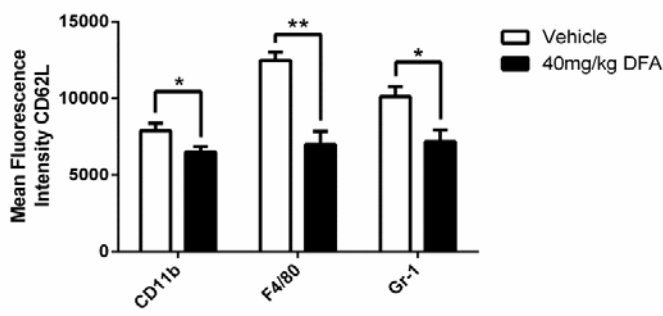


Figure 1. L-selectin expression on CD11b+, F4/80+, and Gr-1+ leukocytes from plasma was decreased 1 day post-DFA administration. N=5/ group. One-way ANOVA followed by Dunnett's post-hoc. *= $p < 0.05$, **= $p < 0.01$

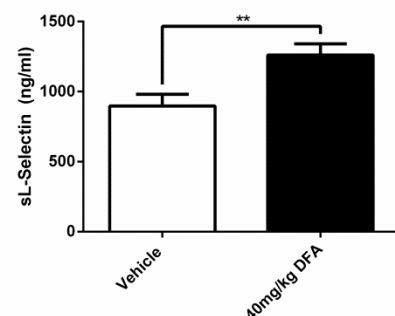


Figure 2. Soluble L-selectin from plasma is increased 1 day post-DFA administration. N=5/group. One-tailed t-test. **= $p < 0.01$

Using flow cytometry, we identified a significant reduction in L-selectin expression on circulating leukocytes from plasma at 1 day post-SCI/DFA administration (Figure 1). Using ELISA, we identified a significant increase in soluble L-selectin in plasma at the same time point (Figure 2). **Taken together, these data suggest that the L-selectin sheddase activity of DFA in plasma can be monitored by utilizing**

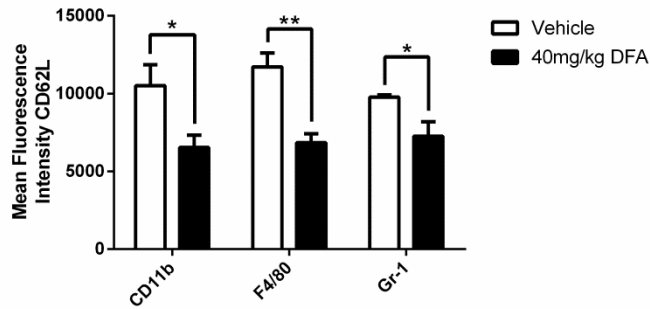


Figure 3. L-selectin expression on CD11b+, F4/80+, and Gr-1+ leukocytes from spinal cord was decreased 1 day post-DFA administration. N=5/ group. One-way ANOVA followed by Dunnett's post-hoc. *= $p<0.05$, **= $p<0.01$

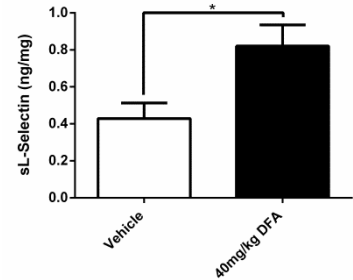


Figure 4. Soluble L-selectin from spinal cord is increased 1 day post-DFA administration. N=5/group. One-tailed t-test. *= $p<0.05$

either flow cytometry to observe reductions in L-selectin expression in leukocytes or ELISA to observe increases in soluble L-selectin that has been shed by circulating leukocytes.

Using flow cytometry, we identified a significant reduction in L-selectin expression on leukocytes from the spinal cord at 1 day post-SCI/DFA administration (Figure 1). Using ELISA, we identified a significant increase in soluble L-selectin in the spinal cord at the same time point (Figure 2). **Taken together, these data suggest that the L-selectin sheddase activity of DFA in the spinal cord can be monitored by utilizing**

either flow cytometry to observe reductions in L-selectin expression in leukocytes or ELISA to observe increases in soluble L-selectin that has been shed by circulating leukocytes.

In summary, these data demonstrate the feasibility of utilizing ELISA to monitor the L-selectin sheddase activity of increasing doses of DFA in both plasma and spinal cord. This methodology was utilized to complete Task 1b.

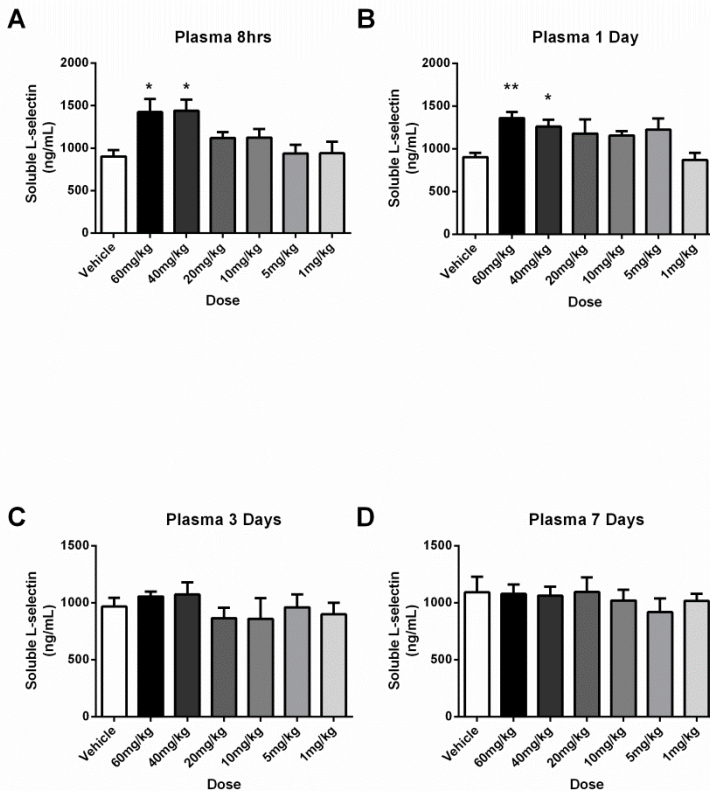


Figure 5: Quantification of soluble L-selectin in plasma from SCI mice at 8 hours, 1 day, 3 days, and 7 days post-DFA administration. N=5/ group. One-way ANOVA followed by Dunnett's post-hoc. *= $p<0.05$, **= $p<0.01$

To assess the L-selectin sheddase activity of increasing doses of DFA, male C57BL/6 mice were subjected to a 2g weight dropped 7.5 cm onto the exposed spinal cord at the thoracic 9 vertebral level (mild injury). DFA (60mg/kg, 40mg/kg, 20mg/kg, 10mg/kg, 5mg/kg, or 1mg/kg) or vehicle (PBS) was administered immediately following spinal cord injury (SCI). L-selectin sheddase activity was quantified by utilizing an enzyme-linked immunosorbent assay (ELISA) that measures the amount of soluble L-selectin in plasma and spinal cord tissue (n=5/group) collected at 8 hours, 1 day, 3 days, and 7 days following SCI/DFA administration. Data from plasma samples is summarized in Figure 5 and data from spinal cord tissue is summarized in Figure 6.

Significance for all data was determined using a one-way ANOVA followed by a Dunnett's post-hoc test and was defined as $p<0.05$.

We identified a significant increase in the levels of soluble L-selectin in the plasma of mice receiving 60mg/kg and 40mg/kg DFA at 8 hours (Figure 5A) and

1 day (Figure 1B) post-injury/administration. By 3 days (Figure 5C), this difference had returned to baseline and continued to be non-significant at 7 days (Figure 5D). The increased levels of soluble L-selectin are indicative of increased L-selectin sheddase activity and suggest that the 60mg/kg and 40mg/kg DFA doses were potent within the first 24 hours of administration. No other doses showed a significant alteration to the soluble levels of L-selectin, suggesting a lack of potency.

We also identified a significant increase in the levels of soluble L-selectin in the spinal cords of mice receiving 60mg/kg and 40mg/kg DFA at 8 hours (Figure 6A) and 1 day (Figure 6B) post-injury/administration. As with the plasma data, this difference had returned to baseline at 3 days (Figure 6C) and was still non-significant at 7 days (Figure 6D). These data corroborate the data from the plasma and furthermore indicate that the L-selectin sheddase activity of 60mg/kg and 40mg/kg DFA is occurring at the target site of interest: the injured spinal cord. As with the plasma data, lower doses of DFA exhibited no significant changes to L-selectin levels in the injured spinal cord.

Taken together, these data suggest that 40mg/kg and 60mg/kg doses of DFA are the minimum effective doses required for L-selectin sheddase activity.

Task 1c. Use a similar dosing strategy and compare neurologic recovery in spinal cord injured mice treated with DFA or vehicle immediately after spinal cord injury (months 10-12).

Male C57BL/6 mice were subjected to a 2g weight dropped 7.5 cm onto the exposed spinal cord at the thoracic 9 vertebral level (. DFA (40mg/kg or 60mg/kg) or vehicle was administered immediately following SCI (n=15/group), using a randomized, blinded design. 5 mice from the vehicle group, 3 mice from the 40mg/kg group, and 3 mice from the 60mg/kg were lost due to surgical/health complications prior to the completion of the study and have been excluded from all subsequent analyses. Mice were weighed prior to injury, followed by 1 day, 3 days, and weekly post-injury (Figure 7). Neurologic recovery was measured using the Basso mouse scale (BMS), where 0 indicates complete hind-limb paralysis and 9 indicates normal locomotion. Testing was performed weekly for a period of 6 weeks post-injury (Figure 8). Terminal assessments of coordination and gait analysis were performed 6 weeks post-injury utilizing gridwalking (Figure 9) and CatWalk kinematic gait analysis (Figure 10). Finally urologic function was assessed by awake cystometry and bladder weight (Figure 11).

Mice were weighed prior to injury, 1 and 3 days post-injury, and then weekly thereafter (Figure 7). A two-way repeated measures ANOVA demonstrated a significant effect for time ($p < 0.0001$), but no effect for treatment ($p > 0.05$). **These data demonstrate that DFA administration did not adversely affect animal weight and overall health.**

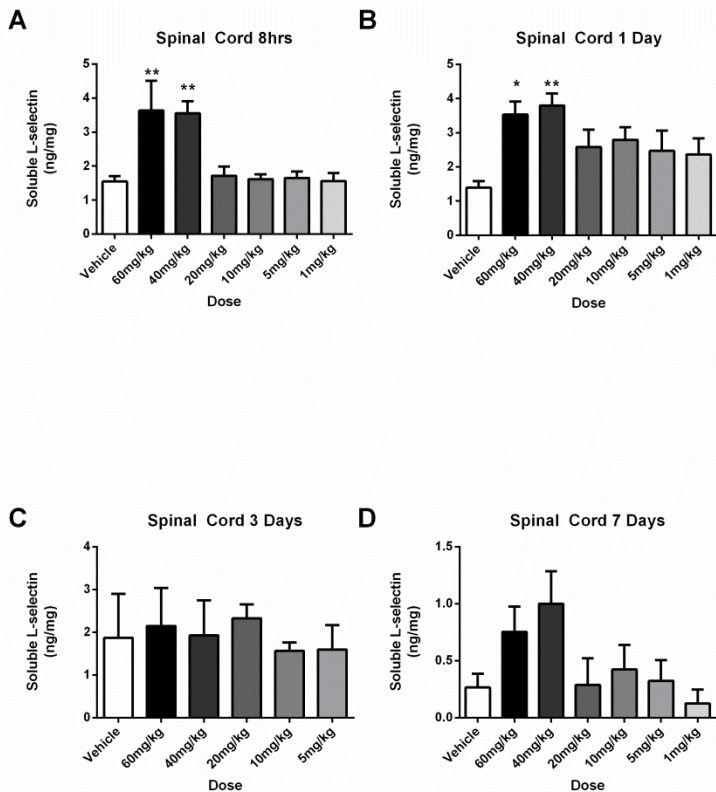


Figure 6: Quantification of soluble L-selectin in the spinal cords from SCI mice at 8 hours, 1 day, 3 days, and 7 days post-DFA administration. N=5/ group. One-way ANOVA followed by Dunnett's post-hoc. *= $p < 0.05$, **= $p < 0.01$

vehicle was administered immediately following SCI (n=15/group), using a randomized, blinded design. 5 mice from the vehicle group, 3 mice from the 40mg/kg group, and 3 mice from the 60mg/kg were lost due to surgical/health complications prior to the completion of the study and have been excluded from all subsequent analyses. Mice were weighed prior to injury, followed by 1 day, 3 days, and weekly post-injury (Figure 7). Neurologic recovery was measured using the Basso mouse scale (BMS), where 0 indicates complete hind-limb paralysis and 9 indicates normal locomotion.

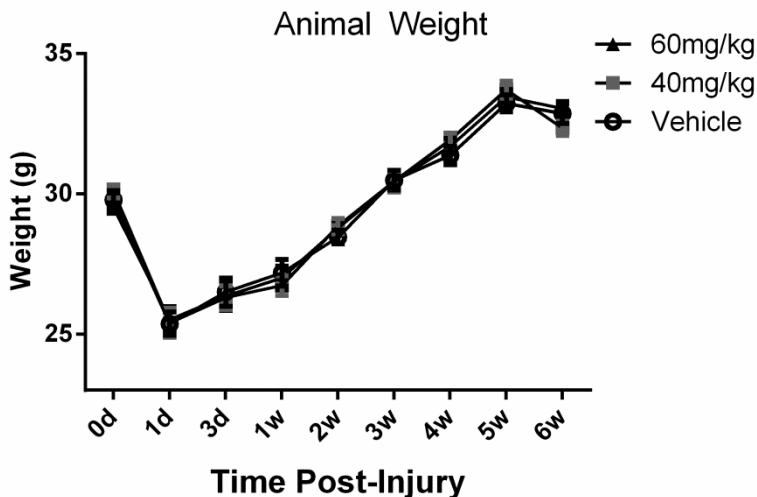
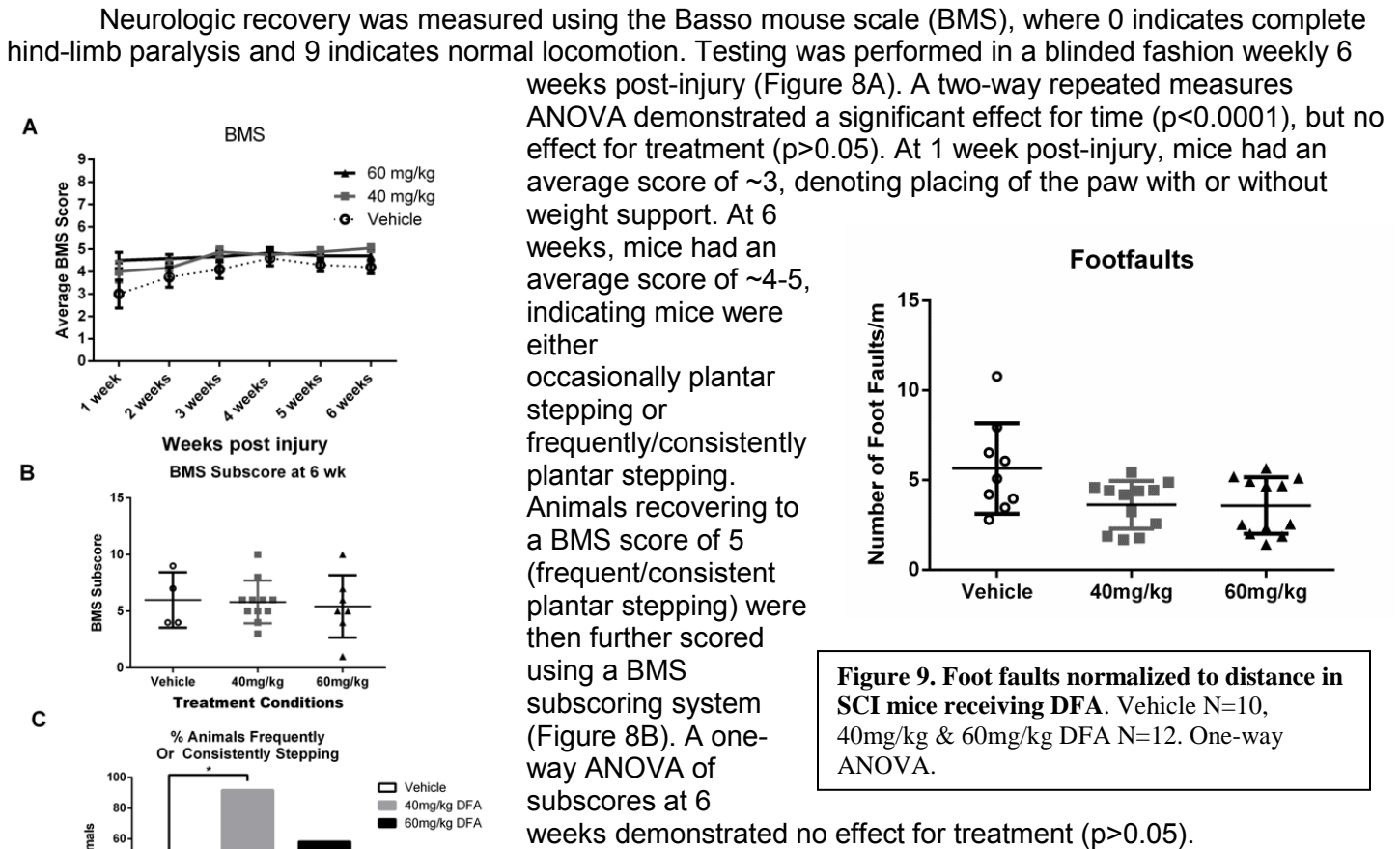
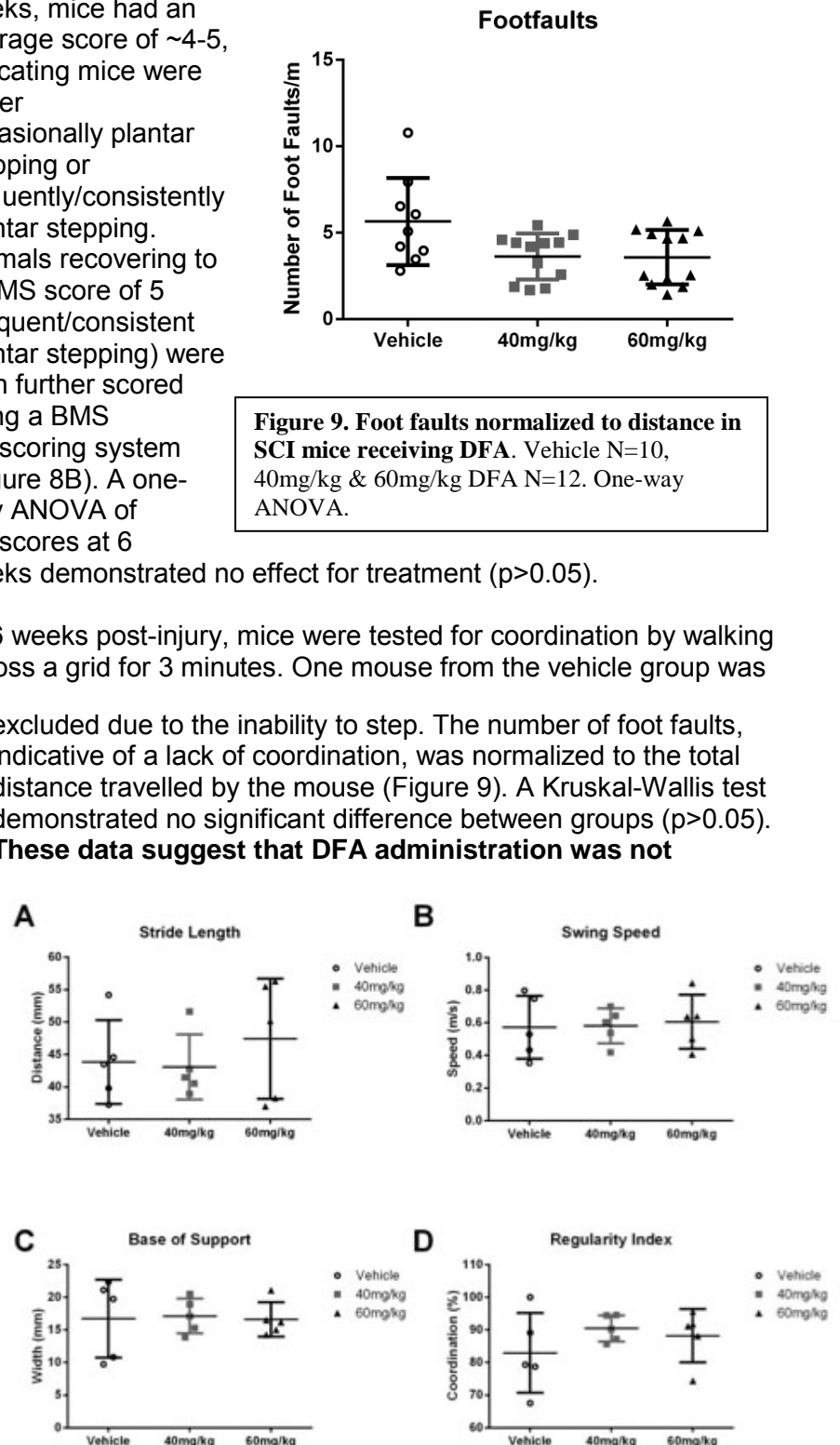


Figure 7: Weight of mice following SCI & DFA administration. Vehicle N=10, 40mg/kg & 60mg/kg DFA N=12. Two-way repeated measures ANOVA.



influencing the overall coordination of mice in this injury paradigm.

Finally, kinematic gait analysis was performed on a small cohort of mice at 6 weeks with CatWalk software. Mice were run across a glass walkway, while a high-speed camera recorded their movements. One mouse from the vehicle group was excluded due to the inability to step. 3 runs were recorded per animal and all 33 animals (Vehicle N=9, 40mg/kg DFA N=12, 60mg/kg DFA N=12) were recorded. Currently, only a subset ($n=5$ from each group) has been analyzed, with the remaining analysis in progress. CatWalk software was utilized to analyze 4 hindlimb



parameters selected a priori: stride length, swing speed, base of support, and regularity index (Figure 10). Stride length and swing speed of the hindlimbs are decreased by SCI, and mice with SCI have a larger hindlimb base of support to compensate for increased trunk instability. Regularity index expresses the number of normal step sequence patterns relative to the total number of paw placements. Mice that have perfect scores of 100% display no deficits in step sequence patterns, whereas those with SCI-induced deficits will have scores <100%. One-way ANOVA for each measure demonstrated no significant differences between groups ($p>0.05$). However, it should be emphasized that statistical power is limited when only assessing a subset of total animals, as visualized by the relatively large error bars. Subsequent analyses utilizing the entire cohort will increase statistical power and may result in detection of drug-dependent effects. **Currently, the kinematic gait of a subset of SCI mice is not influenced by DFA administration in this injury paradigm.**

It is noteworthy that the overall recovery seen in the above study is characteristic of a mild, not a moderate level of SCI. Our objective was to produce a moderate level of injury. However, despite using a setting on the injury device that has historically produced that level of injury, we discovered that mice showed a rapid recovery within the first 1-2 weeks post-injury, a characteristic that is not seen after a moderate level of injury and is consistent with a mild SCI. (We are currently re-titrating the injury device so as to avoid this issue

in any subsequent experiments). A model of mild injury severity is challenging as there is risk of a ceiling effect, whereby treatment will exert no benefit. That is, as animals quickly reach a ceiling of recovery and plateau, the ability to detect drug-dependent effects on locomotor recovery is reduced. Equally importantly, the BMS is an ordinal scale. As such, differences between grades are not linear and in certain cases fail to identify clinically relevant changes. For example, a score of 4 delineates occasional plantar stepping whereas a score of 5 is assigned to frequent/consistent plantar steppers. Such a change in mobility has strong clinical relevance that is not captured by this unweighted scoring system. Given these limitations, we sought to classify mice based on the ability to frequently/consistently plantar step as a method of assessing this portion of the BMS scale (Figure 8C). Frequent/consistent plantar stepping was observed in 40% of vehicle mice, 92% of 40mg/kg DFA mice, and 58% of 60mg/kg DFA mice. Chi square frequency analysis with fisher's exact test demonstrated a significant effect between mice receiving vehicle and 40mg/kg DFA ($p<0.05$). No significant differences were found between vehicle and 60mg/kg DFA or 40mg/kg DFA and 60mg/kg DFA ($p>0.05$). **Together, these data demonstrate that those mice with mild SCIs and receiving 40mg/kg DFA, showed significant plantar stepping relative to the vehicle controls. These findings, together with previous data showing efficacy after a moderate level of SCI, when 40mg/kg DFA was likewise given immediately after SCI, serves to validate DFA across two levels of injury. These collective findings set the stage for testing the therapeutic window after a moderate level of injury and to determine if DFA confers neuroprotection after a severe SCI.**

Finally, in anticipation of future aims and tasks, we sought to evaluate bladder function in DFA-treated mice by awake cystometry in a subset of mice ($n=9$ /group). One mouse from the 40mg/kg DFA group was euthanized due to surgical complications/health issues prior to cystometry. Consistent with this being a mild injury paradigm, bladder functional recovery may have reached a ceiling effect, similar to the

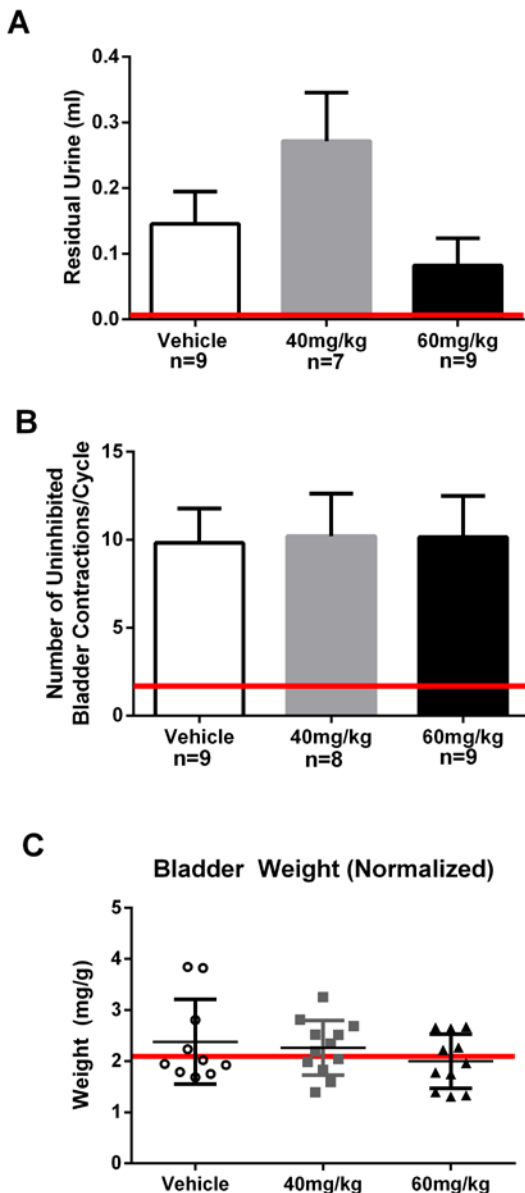


Figure 11. Urological function in SCI mice receiving DFA. A) Residual urine in mice. Vehicle N=9, 40mg/kg DFA N=8, 60mg/kg DFA N=9. Kruskal-Wallis test. Red line indicates uninjured baseline. B) Number of uninhibited bladder contractions/voiding cycle in mice. Vehicle N=9, 40mg/kg DFA N=8, 60mg/kg DFA N=9. Kruskal-Wallis test. Red line indicates uninjured baseline. C) Bladder weight normalized to total body weight. Vehicle N=10, 40mg/kg & 60mg/kg DFA N=12. One-way ANOVA. Red line indicates uninjured baseline.

observations of locomotor recovery. When cystometry was performed at the end of the study, 29/34 (~85%) of mice exhibited partial or complete voluntary bladder voiding during daily animal care. The volume of residual urine (Figure 12A) and the number of uninhibited bladder contractions/voiding cycle (Figure 12B) for all mice have been calculated, and the remaining cystometry data analysis is currently in progress. A Kruskal-Wallis test for each measure demonstrated no significant differences for the volume of residual urine or number of uninhibited bladder contractions/voiding cycle between groups ($p>0.05$). Following euthanasia, bladders were removed, weighed, and normalized to total bodyweight (Figure 11C). One-way ANOVA demonstrated no significant differences on normalized bladder weights between groups ($p>0.05$). Following SCI, bladder dyssynergia typically results in alterations to the levels of smooth/skeletal muscle, as well as connective tissue matrices, leading to a thickening of the bladder wall. However, all SCI mice in this study, regardless of treatment, demonstrated normalized bladder weights comparable to historical uninjured controls. Therefore, any potential DFA-dependent effects on bladder recovery may have been obscured by this degree of baseline recovery. **Taken together, these data suggest that urologic function is not being altered by DFA in this injury paradigm. However, the ability to detect DFA-dependent effects on urological function may be increased in a more moderate or severe SCI paradigm that has a wider range of recovery.**

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated the feasibility of utilizing ELISA to monitor L-selectin sheddase activity
- Identified 40mg/kg & 60 mg/kg DFA as minimal effective doses required for L-selectin sheddase activity
- Demonstrated immediate administration of 40mg/kg following mild SCI results in an increase of frequently/consistently stepping mice
- Established fine motor and kinematic gait analysis protocols for future aims utilizing moderate/severe SCI paradigm
- Established urological function protocols for future aims utilizing moderate/severe SCI paradigm

REPORTABLE OUTCOMES

None

CONCLUSION

- 40mg/kg DFA is the minimal effective dose to induce L-selectin shedding in the plasma and spinal cord following SCI
- Immediate administration of 40mg/kg improves the frequency of stepping in mice with mild SCI

REFERENCES

None

APPENDICES

None

SUPPORTING DATA

None